American Journal of Medical Genetics 63:198–202 (1996)

Methylamine Accumulation in Cultured Cells as a Measure of the Aqueous Storage Compartment in the Laboratory Diagnosis of Genetic Lysosomal Diseases

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Intracellular accumulation of the lysosomotropic compound [^{14}C]methylamine was used to estimate the size of the lysosomal compartment in fibroblasts cultured from patients with a variety of lysosomal storage diseases. In previous work from our laboratory, it was shown that methylamine accumulation was significantly increased in diseases with infantile or juvenile onset and storage of predominantly water-soluble material such as in the mucopolysaccharidoses, mucolipidoses, and oligosaccharidoses. In the present study, methylamine incorporation was abnormally increased in cells from patients with glycogenosis type II and with Niemann-Pick type C disease, whereas it was normal in other sphingolipidoses and in the late-infantile and juvenile forms of neuronal ceroid lipofuscinoses.

The methylamine test was also checked regarding its potential use for prenatal diagnostic testing. In model systems with cultured amniotic or chorionic villus cells, lysosomal storage was experimentally induced by the cathepsin inhibitor leupeptin and was readily detected when compared to untreated controls. Cultured amniotic cells from a fetus with mucopolysaccharidosis II were found to incorporate significantly higher amounts of [^{14}C]methylamine than the normal controls.

The results indicate that the methylamine accumulation method is an additional tool in the diagnosis and prenatal diagnosis of lysosomal diseases with abnormal storage of water-soluble material. © 1996 Wiley-Liss, Inc.

KEY WORDS: diagnosis, lysosomal disease, mucopolysaccharidosis, mucolipidosis, oligosaccharidosis, glycogenosis II, Niemann-Pick type C, neuronal ceroid lipofuscinosis

INTRODUCTION

Among the inborn errors of metabolism, the lysosomal storage diseases are a prominent group of about 40 disorders. Owing to the genetic deficiency of lysosomal enzymes, activator proteins or transporter proteins, material to be degraded or transported accumulates excessively within lysosomes, leading to the enlargement of the organelles, of cells, and whole organs. The storage process may result in severe disease, with clinical symptoms ranging from a "coarse" facial appearance, skeletal abnormalities, and seizures to progressive mental and motor retardation and early death. According to the chemical nature of the storage material, the diseases are grouped as sphingolipidoses, mucopolysaccharidoses, mucolipidoses, oligosaccharidoses, or others [Neufeld, 1991; Scriver et al., 1995; Spranger and Wiedemann, 1970]. A further group where the lysosomal compartment is involved in lipopigment deposition encompasses the neuronal ceroid lipofuscinoses [Kohlschütter et al., 1993]. However, in this group the biochemical basis of the storage process is mostly unresolved.

The diagnosis of a lysosomal disorder requires biochemical tests that demonstrate an enzyme or other protein deficiency, or directly identify a mutant gene. Apart from such specialized procedures, a screening as-

Received for publication January 4, 1996.

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Dedicated to Jürgen W. Spranger on the occasion of his 65th birthday with admiration and best wishes.

say for general lysosomal storage would be a valuable additional tool. We have developed such a method by measuring the accumulation of radioactive methylamine in cultured skin fibroblasts. Methylamine when added to the culture medium, is highly membrane-permeable in its unprotonated form at neutral pH, but is trapped as ionic species (pK_a of 10.7) in the acidic lysosomal compartment [Hollemans et al., 1981]. As the lysosomal pH was found unaltered in the patients' fibroblasts, methylamine accumulation was considered to be directly proportional to the size of the watery phase of the lysosomal compartment. Indeed, all cell lines from patients with infantile or juvenile forms of mucopolysaccharidoses, mucolipidoses, and oligosaccharidoses showed markedly increased radioactivity compared with the normal range of controls; however, in cells from patients with accumulation of lipidic material as in sphingolipidoses, methylamine accumulation was hardly increased [Kopitz et al., 1994].

The present study was undertaken to evaluate the diagnostic potential of the method with regard to diseases not previously tested, and to lysosomal disorders where the metabolic basis is not well defined and a biochemical test parameter is therefore not readily available. In addition, the methylamine incorporation method was checked regarding its possible use for prenatal dignostic testing.

MATERIALS AND METHODS

Chemicals

[^{14}C]Methylamine hydrochloride with a specific radioactivity of 1.9–2.2 GBq/mmol was purchased from Amersham-Buchler (Braunschweig, Germany). Leupeptin hemisulfate salt was obtained from Sigma (Munich, Germany).

Cell Culture

Skin fibroblasts, chorionic villus cells, and amniotic cells from patients and control subjects were cultured in Eagle's MEM with 10% fetal calf serum, non-essential amino acids, and antibiotics in an atmosphere of 95% air and 5% CO_2 [Cantz et al., 1972]. Patients had been diagnosed on the basis of clinical data and, whenever possible, biochemical investigations.

Intracellular [^{14}C]methylamine Accumulation

Intracellular [^{14}C]methylamine accumulation was determined essentially as described [Kopitz et al., 1994]. Briefly, confluent cultures in 25 cm^2 plastic tissue culture flasks were incubated for 3 h at 37°C with 5 ml culture medium containing 6.9 KBq [^{14}C]methylamine/ml and 50 mM HEPES, pH 7.4. Then cells were washed with 3 × 5 ml 0.9% NaCl, harvested by trypsinization, washed again, suspended in 1 ml distilled water, homogenized by sonication, and aliquots used for protein and radioactivity determinations.

During the whole procedure a pH above 8 had to be avoided for all solutions containing [^{14}C]methylamine which might become volatile under such conditions.

RESULTS

The methylamine incorporation was examined in fibroblasts cultured from the skin of patients with various types of Niemann-Pick disease, with glycogenosis type II, and with different forms of neuronal ceroid lipofuscinoses (Batten disease).

In Niemann-Pick disease, the response to [^{14}C]methylamine loading differed according to disease type: whereas fibroblasts from type A and type B exhibited a normal pattern, fibroblasts from all six type C patients showed a markedly increased accumulation of the radioactive amine, indicating an abnormal expansion of the watery phase of the lysosomal compartment (Fig. 1A). The methylamine incorporation was also abnormally increased in all three cell lines from patients with glycogenosis type II (α-glucosidase deficiency, Pompe disease; Fig. 1B). When fibroblast lines from two patients with late-infantile and from four patients with juvenile neuronal ceroid lipofuscinosis were tested, all showed an incorporation of radioactive methylamine that was in the range of normal controls (Fig. 1C). Fibroblast lines from two patients with mucolipidosis IV yielded normal values (results not shown), in contrast to a cell line tested previously that gave an abnormal result [Kopitz et al., 1994].

To evaluate the methylamine test for its use in prenatal diagnosis, we determined normal ranges in cultured amniotic cells and cultured chorionic villus cells from control individuals and tested the possibility of detecting abnormal lysosomal storage by inhibition of lysosomal proteolysis with the cathepsin inhibitor leupeptin. As shown in Figure 2 such artificial storage was easily induced and detected both in amniotic and in chorionic villus cells. Indeed, when amniotic cells from a fetus with mucopolysaccharidosis II (iduronate sulfatase deficiency) were tested in parallel to conventional enzyme and radiosulfate incorporation assays, the [^{14}C]methylamine accumulation was found to be clearly abnormal (Fig. 3).

DISCUSSION

The results of this and our previous study [Kopitz et al., 1994] show that the measurement of [^{14}C]methylamine incorporation in cultured fibroblasts can be used as a diagnostic marker in diseases with excessive storage of water-soluble compounds such as glycosaminoglycans, oligosaccharides, or monosaccharides. However, in fibroblasts from patients with lipid storage diseases such as the sphingolipidoses, the test yielded mostly normal results. This was not unexpected since in the acidic lysosomal milieu, methylamine is predominantly ionized and therefore distributed to the watery phase of the compartment. Thus, methylamine accumulation was significantly increased in fibroblasts from patients with Pompe disease (glycogenosis type II, (α-glucosidase deficiency), indicating abnormal storage of water-soluble glycogen. Indeed, glycogen-filled lysosomes had been demonstrated upon electron microscopy of glycogenosis type II cultured fibroblasts by previous investigators [Hers and de Barsy, 1973]. On the other hand, in fibroblasts from patients with sphingolipidosis of Niemann-Pick disease types A and B, methylamine incorporation was not increased, in keeping with the lipidic nature of the accumulated sphingomyelin. Surprisingly, however, the uptake of radioactive methylamine was abnormally elevated in fibroblasts from

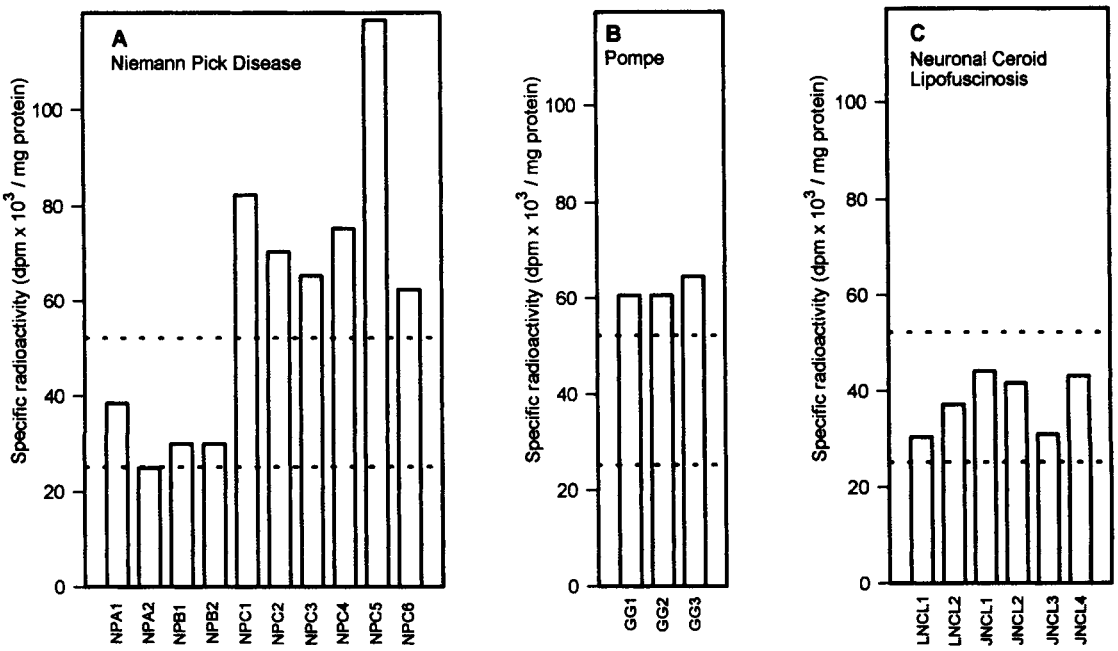

Fig. 1. [^{14}C]Methylamine accumulation in fibroblasts from patients with lysosomal disorders. Fibroblasts were loaded with [^{14}C]methylamine and intracellular radioactivity determined as described under methods. **A:** Fibroblasts from patients with Niemann-Pick disease type A (NPA1-2), type B (NPB1-2), and type C (NPC 1-6). **B:** Fibroblasts from patients with with glycogenosis type II (Gg1-3). **C:** Fibroblasts from patients with late-infantile (LNCL1-2) or juvenile (JNCL1-4) neuronal ceroid lipofuscinosis. The results are the means of at least two independent experiments.
Broken lines (– – –) mark upper and lower limits of normal control range (n = 17; mean ±2 standard deviations).

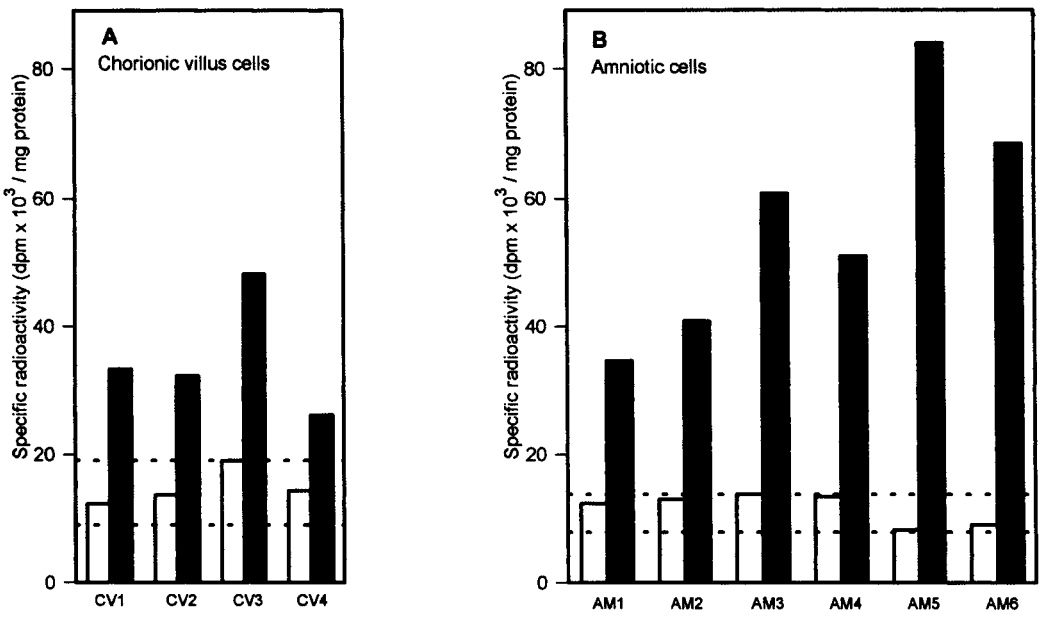

Fig. 2. Effect of leupeptin on [^{14}C]methylamine accumulation in cultured chorionic villus and amniotic cells. 1 mM leupeptin (■) was included in the culture medium of chorionic villus (**A:** CV1-4) and amniotic cells (**B:** AM1-AM6) 4 days before [^{14}C]methylamine accumulation was compared to untreated controls (□).
Broken lines (– – –) indicate normal control range (n = 10 for chorionic villus cells and n = 12 for amniotic cells; minimal/maximal values).

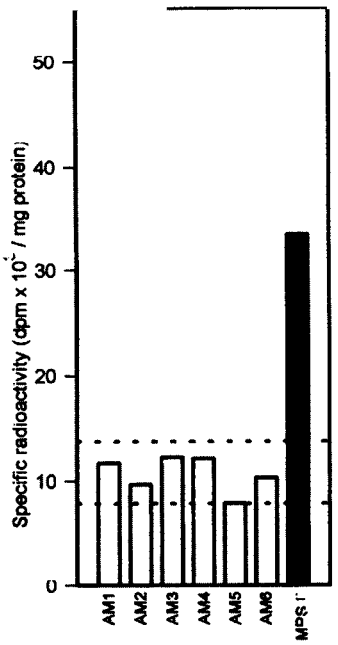

Fig. 3. [^{14}C]Methylamine accumulation in cultured amniotic cells from a fetus with mucopolysaccharidosis II (MPS II; ■) was compared to normal controls (AM1-AM6; (□)).
Broken lines (- - -) indicate normal control range (n = 12; minimal/maximal values).

Niemann-Pick type C patients. This result was consistent in all six cell lines tested and suggests an abnormal lysosomal accumulation of hydrophilic material in addition to the known storage of free cholesterol and other lipids [Pentchev et al., 1995]. Indeed, the finding in Niemann-Pick type C disease of strikingly ballooned neurons and increased water content of the cerebral grey matter was interpreted to be due to osmotic water influx in response to the intracellular accumulation of low molecular weight compounds of a non-lipidic nature [Philippart et al., 1969]. In view of the fact that the laboratory dignosis of Niemann-Pick type C disease can be difficult, the methylamine method could therefore prove to be an additional diagnostic criterion.

In cultured fibroblasts from patients with late-infantile (Jansky-Bielschowsky) and juvenile (Spielmeyer-Vogt) types of neuronal ceroid lipofuscinosis, methylamine loading was in the range of normal controls. In both of these disorders, there is abnormal lysosomal accumulation of subunit c of mitochondrial ATP synthase [Palmer et al., 1992]. As this peptide is very hydrophobic, the situation seems similar to a lipid storage process and may explain the normal methylamine test.

Taken together, the results indicate that the methylamine incorporation test reliably detects lysosomal storage in those diseases, where accumulation of water-soluble material predominates. However, in cells from patients with lipid storage or in adult forms of disease,

Table I. Diagnostic Potential of the [^{14}C]Methylamine Accumulation Test

Disorder	Result of test	Reference
Mucopolysaccharidoses		
Type I	Abnormal	Kopitz et al., 1994
Type II	Abnormal	"
Type IIIA	Abnormal	"
Type IIIB	Abnormal	"
Type VI	Abnormal	"
Type VI late onset	Normal	"
Type VII	Abnormal	"
Mucolipidoses (ML)		
ML II (I-cell disease)	Abnormal	Kopitz et al., 1994
ML III (Pseudo-Hurler polydystrophy)	Abnormal	"
ML IV	Normal / abnormal[a]	This paper
Oligosaccharidoses		
α-Mannosidosis	Abnormal	Kopitz et al., 1994
Sialidosis	Abnormal	"
Sialidosis, late onset	Normal / abnormal[a]	"
Sialic acid storage disease	Abnormal	"
Glycogenosis II (Pompe)	Abnormal	This paper
Sphingolipidoses		
GM1-Ganglipsidosis	Normal	Kopitz et al., 1994
GM2-Gangliosidosis (Sandhoff)	Normal / abnormal[a]	"
Metachromatic Leukodystrophy	Normal	"
Niemann-Pick type A	Normal	This paper
Niemann-Pick type B	Normal	"
Niemann-Pick type C	Abnormal	"
Neuronal ceroid lipofuscinoses (NCL)		
Late-infantile NCL	Normal	This paper
Juvenile NCL	Normal	"

[a]Results have been mostly normal, but occasionally were abnormal.

methylamine incorporation was not abnormally increased. Our diagnostic experience so far is summarized in Table I.

The potential usefulness of the methylamine method for prenatal diagnosis was also examined. In model systems with cultured amniotic or chorionic villus cells, lysosomal storage was experimentally induced by the cathepsin inhibitor leupeptin and was readily detected when compared to untreated controls. Cultured amniotic cells from a fetus diagnosed as having mucopolysaccharidosis II (Hunter disease) were found to incorporate significantly higher amounts of [^{14}C]methylamine than the normal controls. Thus, the results indicate that the methylamine incorporation method can be used as an additional tool in the prenatal diagnosis of lysosomal diseases with abnormal storage of water-soluble material.

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